DETECTION OF *ESCHERICHIA COLI* O157:H7 FROM FOOD BY A MICROPLATE SANDWICH IMMUNOASSAY USING TIME-RESOLVED FLUOROMETRY[1]

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ABSTRACT

Escherichia coli *O157:H7 has been identified as a causative agent in outbreaks of hemorrhagic colitis and hemolytic uremic syndrome involving ground beef and apple cider. A microplate sandwich immunoassay for the detection of* E. coli *O157:H7 from food was developed utilizing time-resolved fluorometry. Biotin-labeled, polyclonal anti-*E. coli *O157:H7 antibodies immobilized on streptavidin-coated microtiter plates were used to capture the bacteria. The bound antigen-antibody complex was then detected using the same antibodies labeled with europium chelates. The effects of capture antibody concentrations, immobilization procedures, and addition of 0.1% Tween 20 to assay buffer were studied during assay development. The detection threshold for the assay developed is 10^3–10^4 CFU/mL. The optimized assay was further tested in ground beef and apple cider samples spiked with* E. coli *O157:H7. The detection limit was < 10 CFU/g of ground beef and ~ 10 CFU/mL of apple cider with 6 h of enrichment.*

[1] Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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INTRODUCTION

Several recent foodborne outbreaks of hemorrhagic colitis and hemolytic uremic syndrome by *Escherichia coli* O157:H7 have heightened the need for rapid and sensitive detection systems for this organism. Undercooked ground beef (Griffin and Tauxe 1991) and unpasteurized apple cider (Besser *et al.* 1993) have been implicated in the majority of outbreaks. Among the developed tests, immunoassays have the advantages of simplicity, speed and high-throughput testing (Johnson *et al.* 1995). Czajka and Batt (1996) developed a solid phase fluorescence capillary immunoassay for the detection of *E. coli* O157:H7 in an antigen down competition format. This assay incorporates a fluorescence cyanine dye, Cy5, in the capillary tube and diode laser/optical sensor system to measure the fluorescence intensity. Seo *et al.* (1998) evaluated immunomagnetic separation with fluorescent antibody-coated beads and flow cytometry for rapid detection of *E. coli* O157:H7 in foods. This system detected 10^3 - 10^4 CFU of *E. coli* O157:H7 in ground beef and could discriminate effectively between *E. coli* O157:H7 and competing natural flora. Fluorescein-conjugated anti-O157 antibody was also used to visualize and enumerate *E. coli* O157:H7 by epifluorescence microscopy (Tortorello and Stewart 1994) or solid-phase laser scanning cytometry (Pyle *et al.* 1999). Microorganisms were either entrapped on a membrane filter or captured by immunomagnetic beads which were then stained with fluorescence dyes, e.g., acridine orange or cyanoditolyl tetrazolium chloride. A fiber-optic biosensor in a sandwich immunoassay format was used to detect *E. coli* O157:H7 by DeMarco *et al.* (1999). This detection system also uses Cy5-labeled polyclonal anti-*E. coli* O157:H7 antibodies to generate specific fluorescent signals between 670 to 710 nm. This approach was able to detect 3 - 30 CFU/mL of *E. coli* O157:H7 in clear liquid samples. However, property variations from fiber to fiber may significantly limit its adoption.

A microplate capture hybridization method for direct detection of verotoxigenic *E. coli* was developed by Cocolin *et al.* (2000). This method employs a biotin-labeled, specific capture probe immobilized to a streptavidin-coated microtiter plate to allow the detection of the polymerase chain reaction (PCR) products by an enzyme-linked immunosorbent assay (ELISA)-based technique.

We have also developed an immunoassay utilizing immunomagnetic separation (IMS) and time-resolved fluorescence (TRF) to detect 10^3 CFU/mL of *E. coli* O157:H7 in apple cider (Yu *et al.* 2002). The TRF procedure has exhibited low detection limits and noise interferences (Degen *et al.* 1999). However, the procedure involving IMS requires the use of magnetic separators and thus adds complications in sample treatment and processing complexities. Thus, in present work we report the development of a rapid and sensitive microplate sandwich immunoassay using time-resolved fluorometry for the

detection of *E. coli* O157:H7 in ground beef and apple cider. The capture antibodies were labeled with biotin to directly immobilize bacteria-antibody complexes to a streptavidin-coated microtiter plate. The bound complexes were detected by Eu-antibody conjugates and evidenced by time-resolved fluorescence. Such a microtiter format should allow multiple samples to be tested simultaneously.

MATERIALS AND METHODS

Preparation of Inocula

E. coli O157:H7 B1409 (Centers for Disease Control and Prevention, Atlanta, GA) and *E. coli* K-12 were stored frozen at -80C in nutrient broth (Oxoid Inc., Ogdensburg, NY) + 0.6% yeast extract (Oxoid) (NB + YE) with 20% glycerol. For all experiments, frozen stocks were inoculated into NB + YE and incubated at 37C for 18 h.

Overnight *E. coli* O157:H7 and K-12 cultures were collected by centrifugation. The pellets were resuspended and serially diluted in phosphate buffered saline, pH 7.4 (PBS; Sigma Chemical Co., St. Louis, MO). Aliquots of 100 μL from appropriate dilutions were plated on Fluorocult *E. coli* O157:H7 agar (EM Science, Gibbstown, NJ) in duplicate and the plates were incubated at 37C for 24 h to determine the inoculum levels.

Microplate Sandwich Immunoassay

The streptavidin assay plates (Becton Dickinson Labware, Bedford, MA) were washed once with washing buffer containing Tris-HCl and Tween 20, pH 7.8 (1:25 dilution; Wallac Oy, Turku, Finland) in an Auto Plate Washer ELx 405 (Bio-tek Instruments, Winooski, VT). One hundred μL of biotin-labeled, goat anti-*E. coli* O157:H7 antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at concentrations of 2-5 μg/mL was added and the immobilization proceeded overnight at 4C (stationary) or 1 h at 25C (with shaking). The antibody was diluted in assay buffer containing Tris-HCl, NaCl, NaN_3, BSA, bovine gamma globulins, Tween 40 and diethylenetriaminepentaacetic acid (Wallac Oy) with or without 0.1% Tween 20 added. After one washing with the washing buffer, 100 μL of serially diluted *E. coli* O157:H7 and/or K-12 in assay buffer with or without Tween 20 were added into each well and incubated for 1 h at 25C on a shaker. After the antigen-antibody binding reaction, the wells were washed once with washing buffer before the addition of 100 μL of filtered europium-labeled antibody (100 ng/mL, PerkinElmer Wallac, Norton, OH) in assay buffer with or without Tween 20 was added and incubated for 1 h at 25C. After the wells were washed three

times with washing buffer, 100 μL of enhancement solution (Wallac Oy) was added to the wells and the microtiter plate was shaken for 5 min. The fluorescence of Eu^{3+}-chelate was measured in a Victor^2 time-resolved fluorometer (PerkinElmer Wallac, Gaithersburg, MD). The schematic representation of the immunoassay was shown in Fig. 1. The limit of sensitivity of the method was determined as the mean value of background + 3 standard deviation (Dahlén *et al.* 1987; Czajka and Batt 1996).

Analysis of Mixed Culture

Overnight cultures of *E. coli* O157:H7 and K-12 were collected by centrifugation and diluted in assay buffer with or without Tween 20 to obtain 10^1 to 10^8 and 10^6 CFU/mL dilutions, respectively. One hundred microliters of the K-12 dilution was mixed in a well with each dilution of O157:H7 with 2 μg/mL of biotinylated antibody immobilized for 1 h at 25C. *E. coli* K-12 was also tested alone to ensure no cross reactivity. The assay was then carried out as described above.

Analysis of Spiked Apple Cider

Overnight cultures of *E. coli* O157:H7 were centrifuged and diluted in apple cider. Two and one-half mL of the apple cider dilutions were added to 22.5 mL modified EC Broth (Difco, Becton Dickinson, Sparks, MD) with 0.02 mg/mL novobiocin (Sigma, St. Louis, MO) to achieve final dilutions of 10^0 to 10^5 CFU/mL. The samples were then incubated for 6 h at 37C with agitation. After enrichment, 100 μL of broth culture was then tested in the microplate sandwich immunoassay using a streptavidin assay plate that had been immobilized with 2 μg/mL of biotinylated antibody to *E. coli* O157:H7 for 1 h at 25C.

Analysis of Spiked Ground Beef

Twenty-five gram portions of ground beef were inoculated with 10^1 - 10^6 *E. coli* O157:H7. Unspiked 25 g samples were also tested for naturally contaminated *E. coli* O157:H7 with Fluorocult *E. coli* O157:H7 agar (EM Science) and served as control. The samples were incubated in Whirl-Pak stomacher bags with filters (Nasco, Fort Atkinson, WI) containing 25 mL of EC broth with novobiocin at 37C for 6 h. After enrichment, samples were homogenized in a Seward Stomacher 400 (Seward Medical, London) before approximately 2 mL aliquots of the broth culture was taken from the filtered side. One hundred microliters of this broth sample was then tested in the microplate sandwich immunoassay with 2 μg/mL of capture antibody. A small aliquot of the filtered broth sample was filtered further through an SMA column

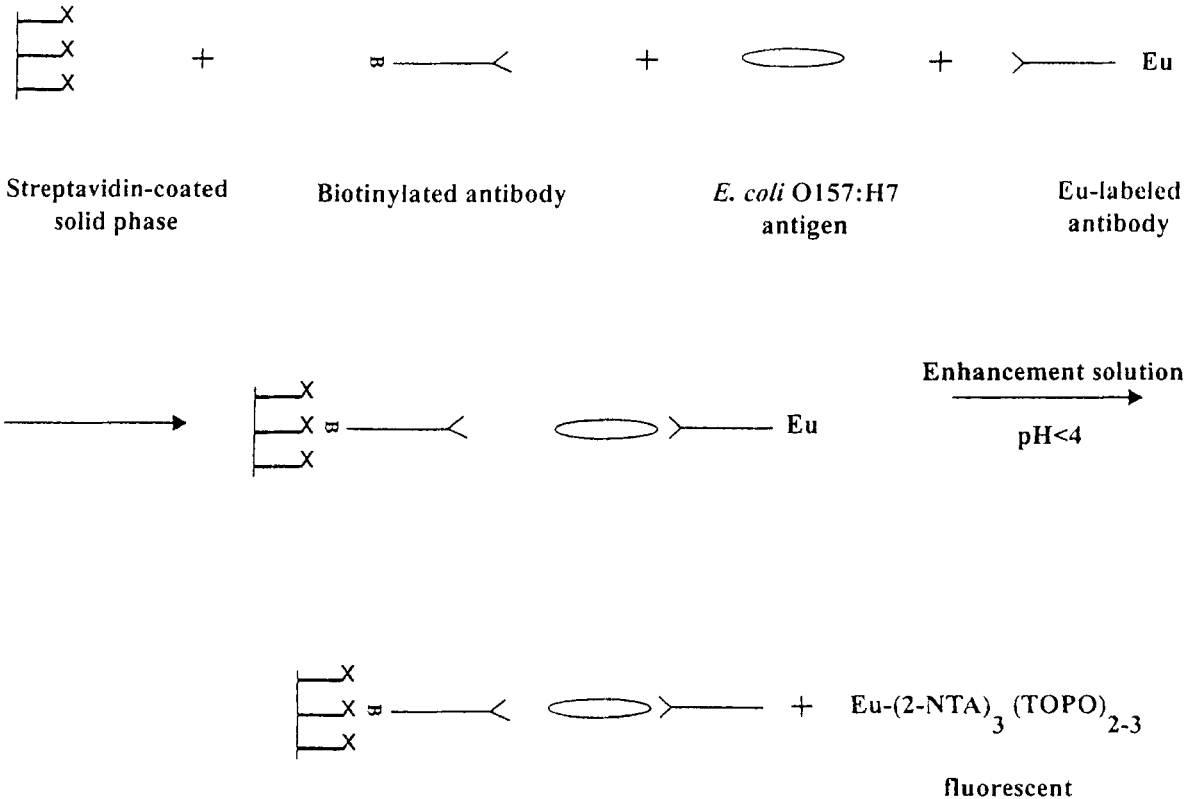

FIG. 1. SCHEMATIC DIAGRAM OF THE MICROPLATE SANDWICH IMMUNOASSAY USING TIME-RESOLVED FLUOROMETRY

(Fisher Scientific, Pittsburgh) to further eliminate large particulates and tested for comparison of fluorescence intensity.

RESULTS

Assay Conditions

Various capture antibody concentrations (2 to 5 μg/mL) were immobilized in the microplate wells for 1 h at 25C or overnight at 4C to determine the effect of antibody concentration on the sensitivity of the assay. The results indicated the immobilization of antibody could be achieved by either approach without significant difference (Fig. 2). Within this antibody concentration range, similar TRF intensities were detected with various cell concentrations (data not shown). Serial dilutions of strain B1409 were tested and the minimum number of cells needed to produce a positive response was 10^3-10^4 CFU/mL.

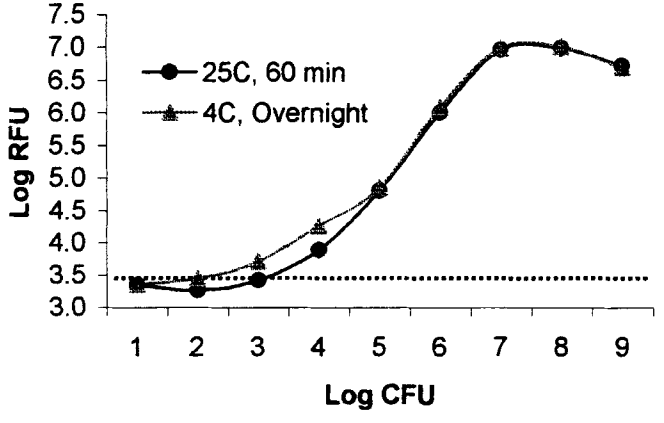

FIG. 2. COMPARISON OF IMMOBILIZATION PROCEDURES USING 2 μg/mL BIOTINYLATED ANTIBODY
The dotted line indicates the limit of sensitivity. The relative percent error between replicates was 18.9%.

Each of the sensitivity assays displays a similar dependence of signal response to bacterial concentration when plotted on a logarithmic scale. A near-linear relationship was observed in the range of 4 to 7 log CFU. As steric hindrances occur as binding sites approach saturation at high concentrations, the

signal intensities started to decline (Fig. 2). The exact reasons for this phenomenon remains to be established.

Mixed Culture and Apple Cider Analysis

Attempts to remove interference with the immunoassay associated with food samples by the addition of Tween 20 to the assay buffer were made. When IMS was used to isolate *E. coli* O157:H7 from food and fecal samples, nonspecific adsorption of other organisms to the IMB was reduced by adding Tween 20 to the washing buffer (Wright *et al.* 1994; Cubbon *et al.* 1996). However, the addition of 0.1% Tween 20 to assay buffer had little effect on the sensitivity of TRF to detect *E. coli* O157:H7 in mixed cultures (Fig. 3) and spiked apple cider (Fig. 4).

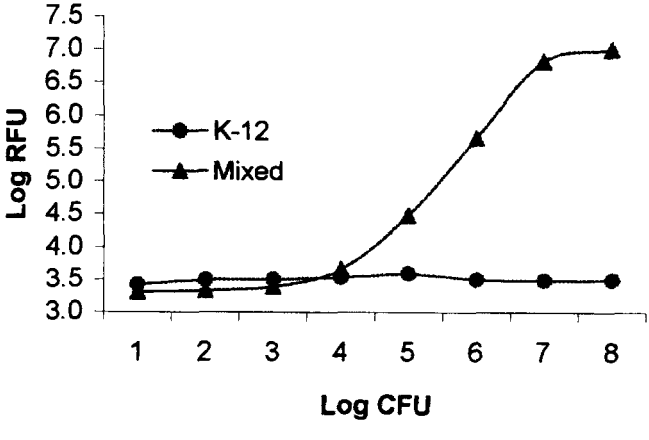

FIG. 3. *E. COLI* K-12 WAS MIXED WITH O157:H7 TO SHOW THE SPECIFICITY OF THE ASSAY

K-12 did not cross react with or inhibit the detection of O157:H7. The relative percent error between replicates was 24% for the mixed cultures and 16.1% for *E. coli* K-12.

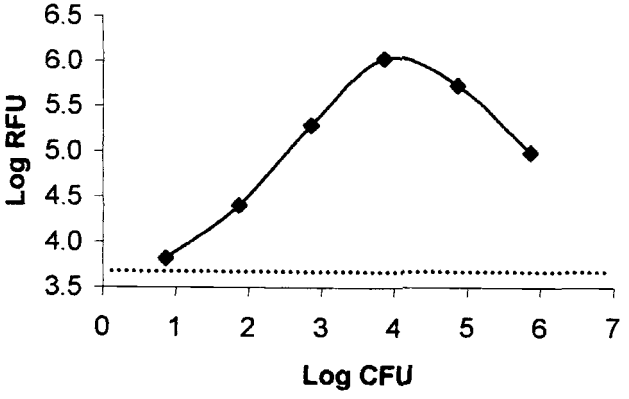

FIG. 4. DETECTION OF *E. COLI* O157:H7 FROM SPIKED APPLE CIDER
The dotted line indicates the limit of sensitivity. A relative percent error of 10.7% was found between replicate points.

Ground Beef Analysis

The presence of normal microflora in ground beef did not appear to cause any significant increase in fluorescence signal (Fig. 5). Again, the addition of Tween 20 to the assay buffer also produced little difference. Moreover, the higher particle content of the samples filtered only once did not appear to have any significant effect on the sensitivity of the assay, as the results were comparable to those obtained when assays were performed on the twice-filtered samples.

DISCUSSION

The application of TRF technology combining with immunomagnetic separation (IMS) to detect pathogenic bacteria in foods has been previously reported (Tu *et al.* 2001). To utilize IMS to concentrate target pathogens, effective magnetic separators are needed. The multiple bead transfer steps involved in the process would certainly add probable errors to the analysis. Thus, despite its convenience, alternatives to IMS are needed.

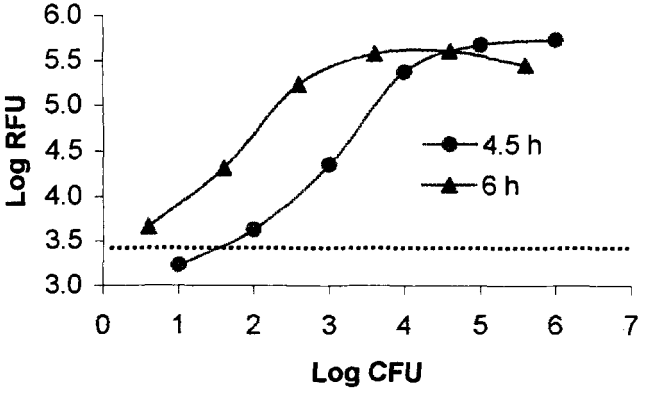

FIG. 5. SPIKED SAMPLES OF GROUND BEEF WERE ENRICHED FOR 4.5 AND 6 H TO COMPARE THE DETECTION LEVEL
After 6 h of enrichment, lower numbers of *E. coli* O157:H7 were able to be detected. Relative percent error was found to be 30% between replicates.

Streptavidin coated microtiter plates have been utilized to detect PCR products of verotoxigenic *E. coli* (Chen *et al.* 1998; Cocolin *et al.* 2000). In present work, polyclonal anti-*E. coli* O157 antibodies were immobilized on microtiter plates using biotin-streptavidin chemistry. This stationary antibody surface was then used to capture and concentrate spiked *E. coli* O157:H7. After a brief enrichment in EC media, very low levels of the bacteria spiked in hamburger (<10 CFU/g) and apple ciders (~ 10 CFU/mL) could be detected. Relative to IMS approach, the microtiter plate method offered a few distinctive advantages. First, the enriched bacterial suspensions were applied directly to the plates and captured bacteria remained in the same wells throughout the entire detection procedure. For high throughput operation, ordinary plate washer could be applied to decrease the sample processing time. The use of microtiter plates to replace IMS, did not change the minimal cross reactivity with nonpathogenic *E. coli* K-12. These results indicated that the reported procedure may be further developed as an effective screening test for *E. coli* O157:H7 in foods.

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